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Inhibitory effect of type 1 collagen gel containing α-elastin on proliferation and migration of vascular smooth muscle and endothelial cells

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The purpose of this study was to investigate *in vitro* the potential effect of type 1 collagen gel containing α-elastin on the proliferation of vascular smooth muscle cells and vascular endothelial cells, and on smooth muscle cell migration. Vascular smooth muscle cell and endothelial cell were cultured in 12-well plates precoated with collagen gels and α-elastin. Cell proliferation rates were measured by monitoring [^{3}H]-thymidine incorporation. After 2, 3 or 4 days of culture, the proliferation rate of both smooth muscle cells and endothelial cells was significantly decreased on collagen gel containing 10 mg/ml α-elastin compared with collagen gel only as control. Smooth muscle cell proliferation on collagen gel containing α-elastin on the 4th day of culture was decreased dose-dependently, e.g. 1 mg/ml of α-elastin (74.8(2.3)% of control, P = n.s.); 5 mg/ml (56.7(2.1)%; $P<0.05$); 10 mg/ml (30.3(3.1)%; $P<0.005$). In the case of cultured endothelial cells, however, [^{3}H]-thymidine incorporation was not decreased significantly in the presence of 5 mg/ml α-elastin (83.1(7.9)%, P = n.s.). After stimulation by platelet-derived growth factor, the smooth muscle cell migration rate on collagen gel containing α-elastin (5 mg/ml) was decreased over time. The area of migration on the 6th day of culture was also significantly decreased dose-dependently in the presence of α-elastin, e.g. 1 mg/ml (72.6(3.4)% of control, $P<0.05$), 5 mg/ml (56.9%(1.5)%; $P<0.05$); 10 mg/ml (37.3(2.7)%; $P<0.0005$). In conclusion, α-elastin inhibited the proliferation and migration of smooth muscle cell in a dose-dependent manner on collagen gel culture, however, at high concentrations of α-elastin (10 mg/ml), the endothelial cell proliferation rate was also inhibited. At 5 mg/ml, α-elastin significantly inhibited smooth muscle cell proliferation and migration but did not significantly inhibit endothelial cell proliferation. Incorporation of collagen gel containing α-elastin into the structure of arterial prosthesis offers the possibility of inhibiting smooth muscle cell hyperplasia without significant effect on endothelial cell formation. © 1997 The International Society for Cardiovascular Surgery.

Keywords: smooth muscle cell, endothelial cell, α-elastin

Intimal hyperplasia caused by excessive growth of smooth muscle cells at the graft–artery interface or site of interventional trauma is a major impediment to the long-term patency of arterial reconstruction [1]. After successful intervention, growth factors stimulate smooth muscle cells to proliferate and migrate, resulting in intimal hyperplasia [2–5]. Innovations aimed at the prevention of intimal hyperplasia include hybrid arterial prostheses made by seeding grafts with vascular endothelial cells [6], and prostheses composed of auto-connective tissue [7], or coated with fibronectin [8] or fibroblast growth

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factor [9, 10]. None of these methods, however, enhances endothelialization, yet prevents excessive myointimal hyperplasia. Several studies have shown that elastin peptides, found adherent to the substratum of the native elastic lamina of arteries, have an inhibitory effect on the proliferation and migration of smooth muscle cells [11–15]. In its natural form, elastin is an insoluble protein [16], but soluble elastin inhibits platelet aggregation induced by collagen [17]. In this study, soluble α-elastin [18–20] was used to investigate the potential effect of type 1 collagen gel containing α-elastin on the proliferation of smooth muscle cells and endothelial cells and migration of smooth muscle cells *in vitro*.

Materials and methods

Cell culture

Smooth muscle cells were prepared from explants of porcine thoracic aorta according to the method of Ross [21], and endothelial cells were obtained from the porcine aortic luminal surface [22]. These cells were cultured at 37°C in a 5% CO_2 atmosphere in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), penicillin (100 units/ml), and streptomycin (100 units/ml). All tissue culture reagents were purchased from Gibco (Grand Island, NY, USA). On microscopic examination, confluent cultures of smooth muscle cells formed characteristic hill-and-valley patterns, whilst endothelial cells formed characteristic cobblestone-like patterns. Smooth muscle cells and endothelial cells were utilized in the third through fifth passages.

Preparation of type 1 collagen gel containing α-elastin

One ml of 10× Hanks' balanced salts solution (Sigma Chemical Co. St. Louis, MO), 100 μl of 100× *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid solution (Fisher Scientific, Fair Lawn, NJ), 800 μl of distilled water and 8 ml of PC-3 type 1 collagen solution (ICN Biomedicals Inc., Costa Mesa, CA) were mixed in a 50-ml conical tube (Fisher Scientific) at 2–4°C respectively. The pH of this solution was adjusted to 7.4 by addition of 7.5% sodium bicarbonate solution. α-Elastin was added to the solution and mixed to produce final concentrations of 1, 5 and 10 mg/ml. One ml of this solution was alloted to each of the 12 wells and incubated for 20 min at 37°C in a 5% CO_2 atmosphere.

Cell proliferation assay

Cell proliferation rate was measured by monitoring [^{3}H]-thymidine incorporation into DNA of cultured smooth muscle cells and endothelial cells. Smooth muscle cells and endothelial cells (4×10^4 cells/well) were cultured in 12-well plates (containing 1 ml/well of type 1 collagen gel+10 mg/ml of α-elastin). Collagen gel-only wells (1 ml) were used as a control. The cells were pulse-labelled with 2 μCi/well of

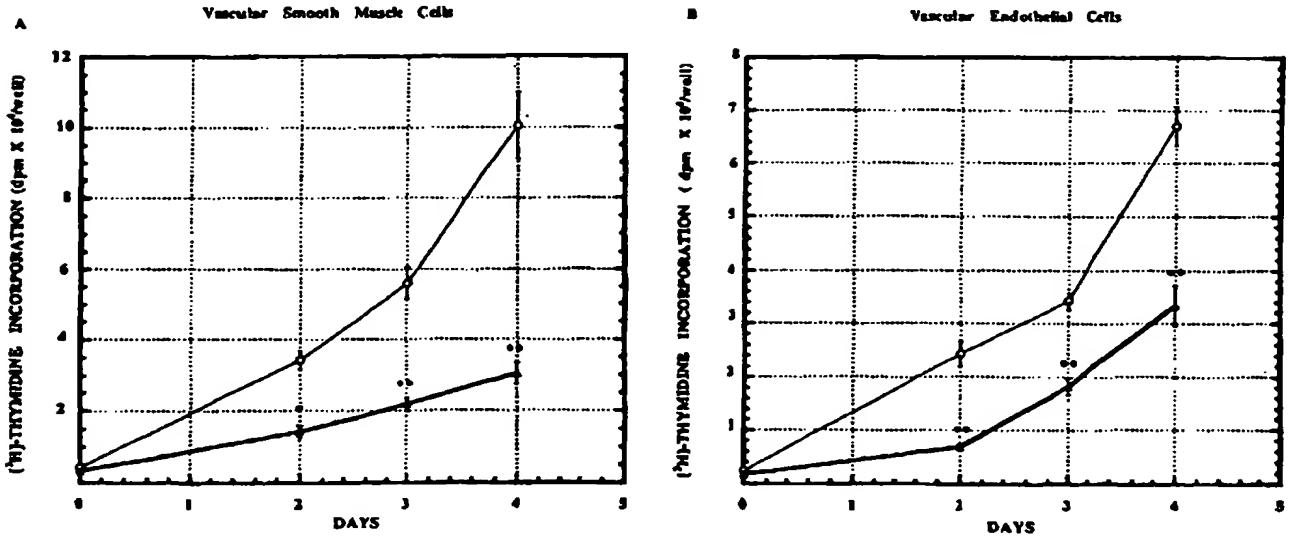

Figure 1 Growth curves of A smooth muscle cells and B endothelial cells on type I collagen gel culture. C, control (type I collagen gel without α-elastin); ▲, type 1 collagen containing 10 mg/ml α-elastin. Results were expressed as mean(s.e.m.). *$P<0.05$. **$P<0.01$. versus control (paired Student's *t*-test).

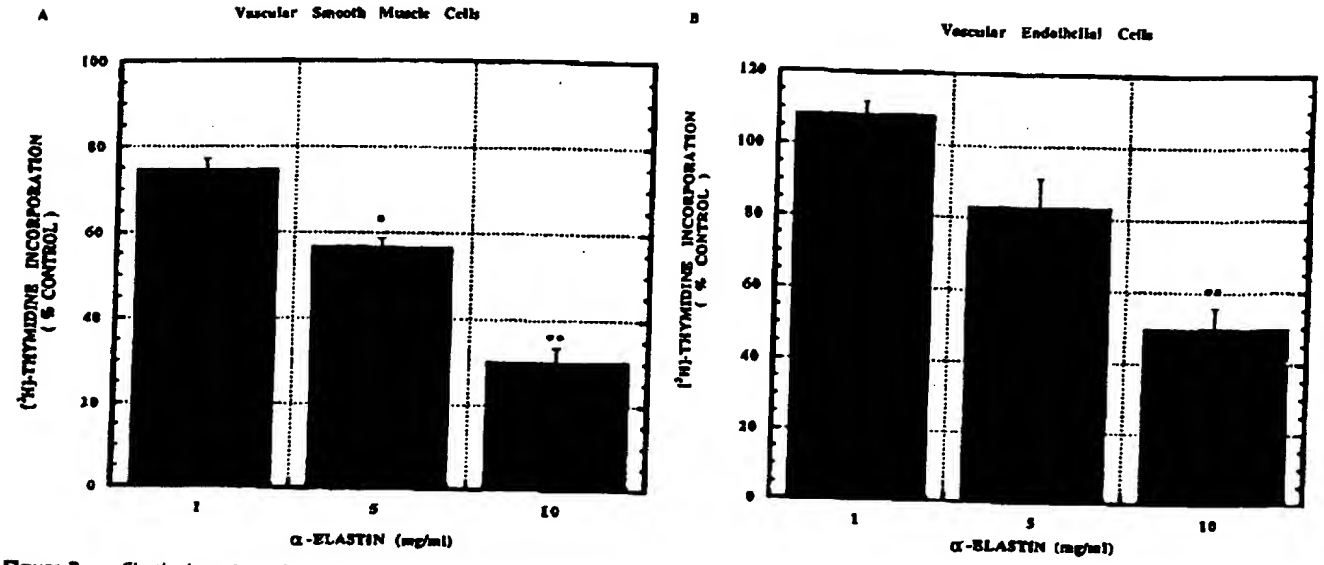

Figure 2 α-Elastin dose-dependent proliferation of A smooth muscle cells and B endothelial cells on the 4th day of culture. [³H]-Thymidine incorporation is presented as % of control (type 1 collagen gel) cultures without α-elastin. Results were expressed as mean(s.e.m.). $^{*}P<0.05$; $^{**}P<0.01$, *versus* control (paired Student's *t*-test).

[³H]-thymidine (64 Ci/mmol; ICN Radiochemicals, Irvine, CA) after 1, 2 or 3 days of culture at 37°C in a 5% CO_2 atmosphere in DMEM with 10% FBS. At 24 h after each pulse-labelling, the cells were harvested from the collagen gel by exposure to 1 ml/well of 0.1% collagenase solution (Gibco) for 10 min at 37°C, after washing three times with ice-cold phosphate buffered saline. The cell suspensions were solubilized in 0.5% sodium dodecyl sulfate and precipitated by 10% cold trichloroacetic acid for 30 min. The precipitates were trapped on a type GF/C glassfiber filter (Fisher Scientific, Pittsburg, PA). Radioactivity incorporated into DNA was determined by liquid scintillation counting of the GF/C filters. At day 0, cells were pulse-labelled with 2 μCi/well of [³H]-thymidine at 37°C for 1 h. On the 3rd day of culture, the cell proliferation rate on collagen gel containing various concentrations of α-elastin (0, 1, 5 or 10 mg/ml) was assayed by the same procedure. All experiments were carried out four times and the results expressed as disintegrations/per min of [³H]-thymidine/well of total DNA, and as percentage of control. After 4 days of culture, cells on 12-well plates were washed twice with phosphate-buffered saline, and fixed for 1 h at 23°C in 10% buffered formalin phosphate (Fisher Scientific), and then stained for 3 min at 23°C using toluidine blue. Cells were then examined by a dissection microscope (Olympus, Tokyo, Japan).

Cell migration assay

Smooth muscle cells (4×10^4 cells/circle) were cultured on a 12-mm circular coverslip (Fisher Scientific). After 3 days of culture, the coverslip was placed at the edge of a well in the 12-well plates (containing 1 ml/well of type 1 collagen gel+5 mg/ml α-elastin). A filter paper (0.6-mm, circular; Schleicher & Schuell, Keene, NH) cut in half and treated with 1 unit/20 μl of platelet-derived growth factor (Porcine Platelet; ICN Biomedicals, Inc. Aurora, OH) was placed opposite the 12-mm coverslip stimulate smooth muscle cell migration.

After 4, 5 or 6 days of culture, smooth muscle cells were stained with toluidine blue as described for the cell proliferation assay. Photographs of dissection microscopic images were taken and the pictures scanned and displayed on a monitor (Microtec International, Inc. Redondo Beach, CA) and Adobe PhotoshopTM2.0 software (Adobe Systems, Inc. Mountain View, CA) for area measurement. Smooth muscle cell migration rate was determined by measuring the area of migration from the 12-mm coverslip by computer analysis (IPLab Spectrum Serious Image Processing for The Macintosh II; Signal Analytics Corporation Vienna, VA).

Migration of smooth muscle cells on collagen gel containing various concentrations of α-elastin (0, 1, 5 or 10 mg/ml) was assayed by the same procedure

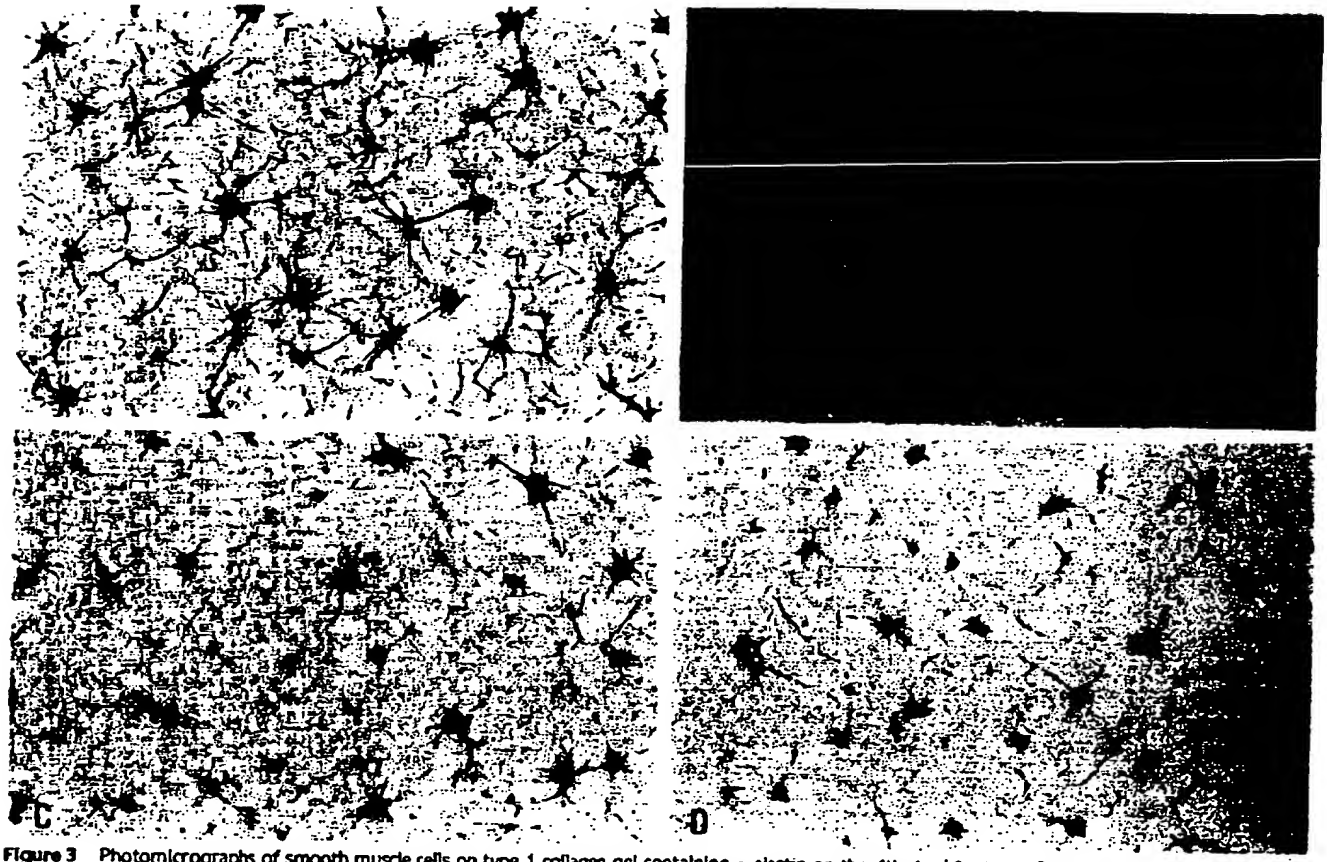

Figure 3 Photomicrographs of smooth muscle cells on type 1 collagen gel containing α-elastin on the 4th day of culture. Smooth muscle cells were stained with toluidine blue. A Control (collagen gel only); B 1 mg/ml α-elastin; C 5 mg/ml α-elastin; D 10 mg/ml α-elastin. In contrast to A, proliferation and network formation (arrows) of smooth muscle cells D are inhibited by increasing dosages of α-elastin. Original magnification: ×45.

on the 6th day of culture. All experiments were carried out four times and the results were expressed as mm² and as percentage of control.

Statistical analysis

Results are expressed as mean(s.e.m.). Significance of statistical differences were assessed with paired Student's *t*-test; statistical significance was accepted when $P<0.05$.

Results

Effect of type 1 collagen gel+α-elastin on smooth muscle cell and endothelial cell proliferation

On collagen gel culture, morphological observation of smooth muscle cell growth showed typical forms resembling that on the glass culture bottle [23]. In the presence of 10 mg/ml α-elastin, [³H]-thymidine incorporation into smooth muscle cells and endothelial cells was significantly inhibited over time (*Figure 1*). Further, in the presence of α-elastin, the proliferation rate of smooth muscle cells on the 4th day of culture was decreased dose-dependently, e.g. 1 mg/ml (74.8(2.3)%, P=n.s.), 5 mg/ml (56.5(2.1)%, $P<0.05$), 10 mg/ml (30.3(3.1)%, $P<0.005$) (*Figure 2*). In the presence of 10 mg/ml α-elastin, endothelial cell proliferation rate was markedly decreased (49.8(5.7)%, $P<0.005$) (*Figure 2B*). However, endothelial cell proliferation was not decreased significantly compared with controls at concentrations of 1 mg/ml (108.4(3.4)%. P=n.s.) or 5 mg/ml (83.1(7.9)%, P=n.s.) (*Figure 2B*). Photomicrography confirmed that proliferation and network formation of smooth muscle cells on collagen gel were reduced dose-dependently in the presence of α-elastin (*Figure 3A–D*). Endothelial cells on col-

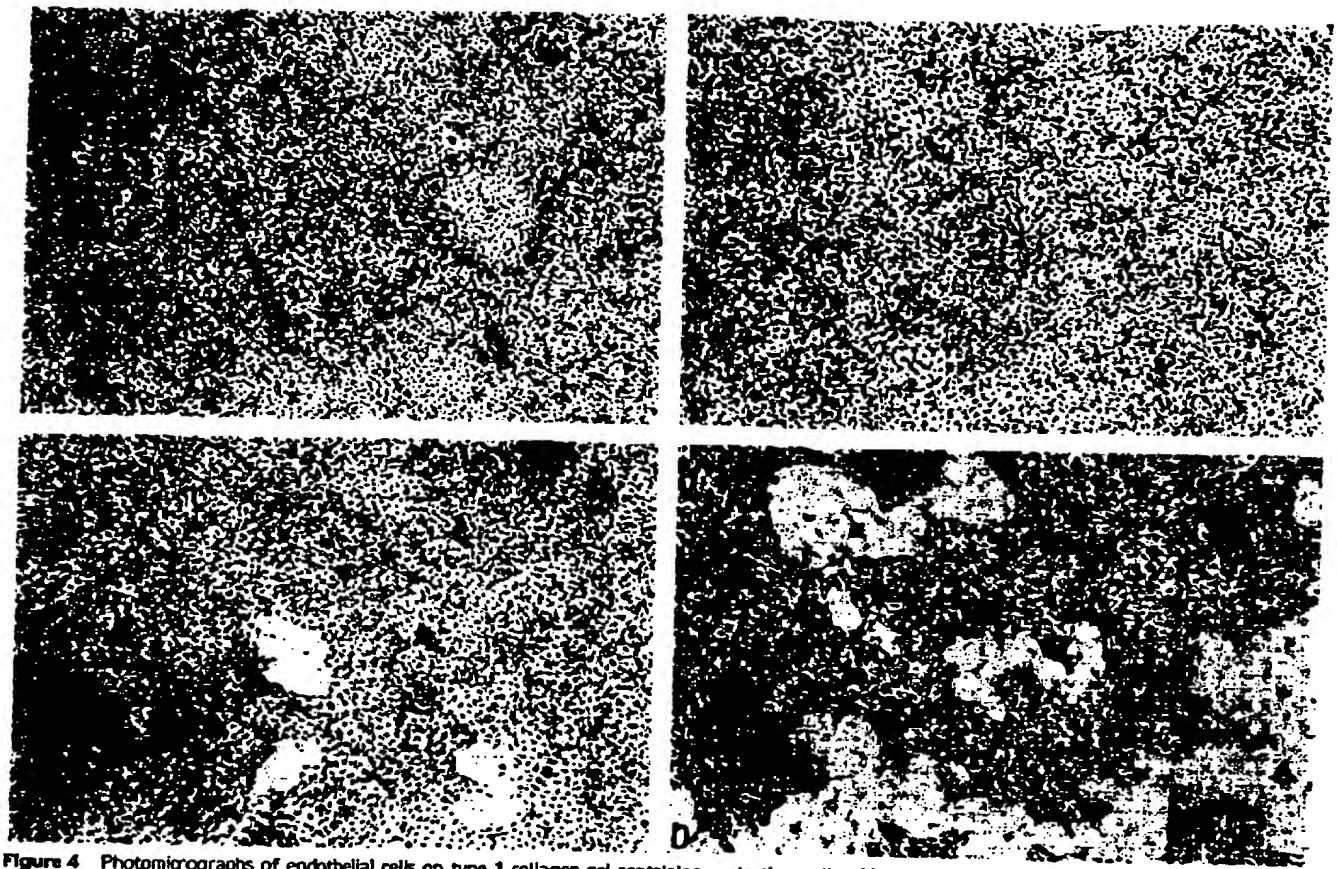

Figure 4 Photomicrographs of endothelial cells on type 1 collagen gel containing α-elastin on the 4th day of culture. Endothelial cells were stained with toluidine blue. A Control (collagen gel only): B 1 mg/ml α-elastin, endothelial cells formed a confluent monolayer with a cobblestone-like appearance; C 5 mg/ml α-elastin, endothelial cells formed an almost complete monolayer; D 10 mg/ml α-elastin, endothelial cells formed an incomplete monolayer. Original magnification, ×45.

lagen gel containing 1 and 5 mg/ml α-elastin formed a monolayer of cobblestone appearance and were confluent on the 4th day of culture (*Figure 4*A–D).

Effect of type 1 collagen gel+α-elastin on smooth muscle cell migration

Migration of smooth muscle cells was stimulated by using graded doses of platelet-derived growth factor on collagen gel as a positive control. Using the same method, the smooth muscle cell migration on collagen gel in the presence of 5 mg/ml α-elastin over time was investigated, as at this dosage, α-elastin significantly inhibited smooth cell proliferation without inhibiting endothelial cell proliferation. The migration of smooth muscle cells on collagen gel containing 5 mg/ml α-elastin was measured between days 4 and 6 of culture, as smooth muscle cells on the 12-mm coverslip were not stimulated by platelet-derived growth factor during the first 3 days of culture. A concentration of 5 mg/ml α-elastin in collagen gel inhibited the migration area of smooth muscle cells over time (*Figure 5*). On the 6th day of culture, various concentrations (1, 5 and 10 mg/ml) of α-elastin significantly, and dose-dependently decreased smooth muscle cell migration, e.g. 1 mg/ml (72.6(3.4)%, $P<0.05$); 5 mg/ml (56.93(1.45)%, $P<0.05$); 10 mg/ml (37.25(2.75)%, $P<0.0005$) (*Figure 6* and *Figure 7*A–D).

Discussion

Injury of the internal elastic lamina of arteries by operation or endovascular methods activates smooth muscle cells in the media by several mechanisms, including secretion of chemoattractants and mitogenic factors [2–5]. Activated smooth muscle cells change their phenotype from the contractile to the synthetic type [24, 25], and then migrate and proliferate in the intima to produce intimal hyperplasia.

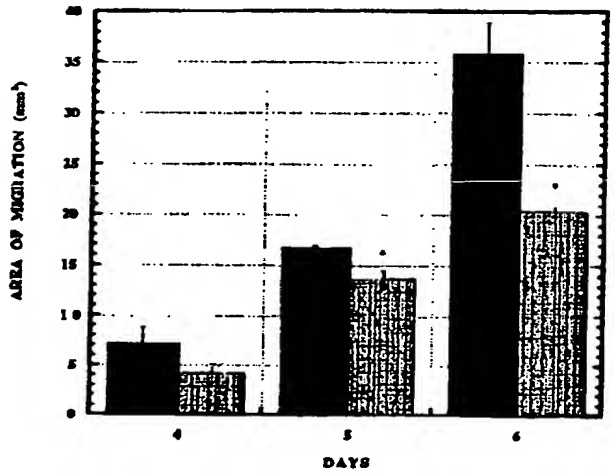

Figure 5 α-Elastin decreases smooth muscle cell migration. Type 1 collagen gel without α-elastin (solid bars) *versus* type 1 collagen gel containing 5 mg/ml α-elastin. Results were expressed as means(s.e.m.). *$P<0.05$ *versus* control (paired Student's *t*-test).

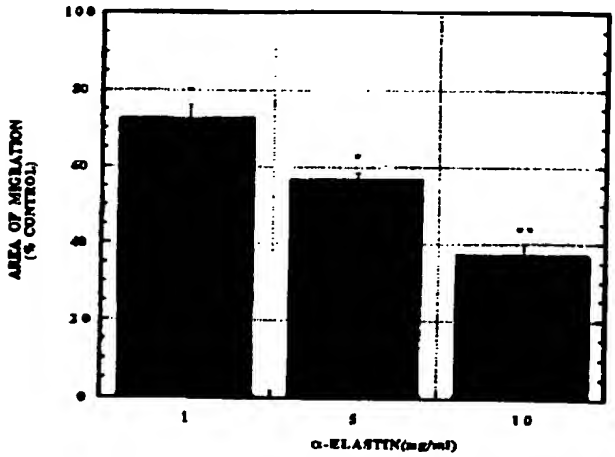

Figure 6 Dose-dependent effect of α-elastin on smooth muscle cell migration on the 6th day of culture. The area of migration is presented as % of control (type 1 collagen gel) cultures without α-elastin. Results were expressed as mean(s.e.m.) *$P<0.05$; **$P<0.0005$, *versus* control (paired Student's *t*-test).

Elastin, an insoluble protein produced specifically by smooth muscle cells in the media of arteries, confers elasticity and supports transportation of metabolic substances in arterial walls [16]. The proelastin 60–70 kDa molecule, after production and release by smooth muscle cells, undergoes crosslinking, and elastin fiber formation is promoted by the enzyme lysyl oxidase [26]. Several studies have reported that the internal elastic lamina of an artery has an inhibitory effect on smooth muscle cell migration [11]. Such inhibition appears to be relatively specific for elastin peptides and for smooth muscle cells, as other matrix components did not impede smooth muscle cell migration. Further, the phenotype of smooth muscle cells, while in the media, is limited to the contractile type by elastin [11–13].

It was hypothesized by the authors that inclusion of an elastin peptide as a constituent of a prosthesis may inhibit intimal hyperplasia. The insoluble native elastin does not readily form solutions in collagen gel; therefore, investigation of the effect of elastin on migration and proliferation of smooth muscle cells *in vitro* is impractical. α-Elastin is a soluble protein purified from bovine neck ligament by a coacervation method [18]. This protein was selected for examination of any effect on smooth muscle cell migration and proliferation. Two previous reports had suggested that the migratory response of smooth muscle cells to chemoattractants is inhibited by soluble elastin peptides in solid phase [13, 27]. Further, soluble elastin also inhibits platelet aggregation induced by collagen [17]. Therefore, the potential inhibitory effect of α-elastin on the growth and migration of smooth muscle cells was investigated, using collagen gel as the solid phase matrix component. Cell proliferation rates were assayed by measuring the incorporation of [^{3}H]-thymidine into DNA of cultured smooth muscle cells and endothelial cells. α-Elastin inhibited smooth muscle cell proliferation in dose-dependent manner (*Figure 2*), while only the highest concentration of α-elastin (10 mg/ml) significantly inhibited endothelial cell proliferation (*Figure 1B*). At a concentration of 5 mg/ml, α-elastin significantly inhibited smooth muscle cell proliferation (*Figure 2A*), but not that of endothelial cells (*Figure 2B*). It should be noted that proliferation was decreased in both endothelial cells and smooth muscle cells, but reached statistical significance only in the latter cell type. However, since these observations were made on relatively small population sizes it is possible that, in studies with larger sample sizes, the effects would not be apparent.

Cell migration assays were performed currently by several methods, including the modified Boyden chamber technique [4], phagocytokinetics [28], and also by measurement of cell migration distance [27, 29]. For measurement of smooth cell migration, a new analytical method was devised that assayed the area of migration by computer analysis. After 3 days of culture, smooth muscle cells stimulated by platelet-derived growth factor began to migrate. Collagen gel containing 5 mg/ml α-elastin significantly inhibited such proliferation and migration of smooth muscle cells with no other significant effect on endothelial cell proliferation. Since these studies were not performed using techniques to block smooth muscle cell proliferation, it is possible that the changes in cell migration reflect to some degree, the concomi-

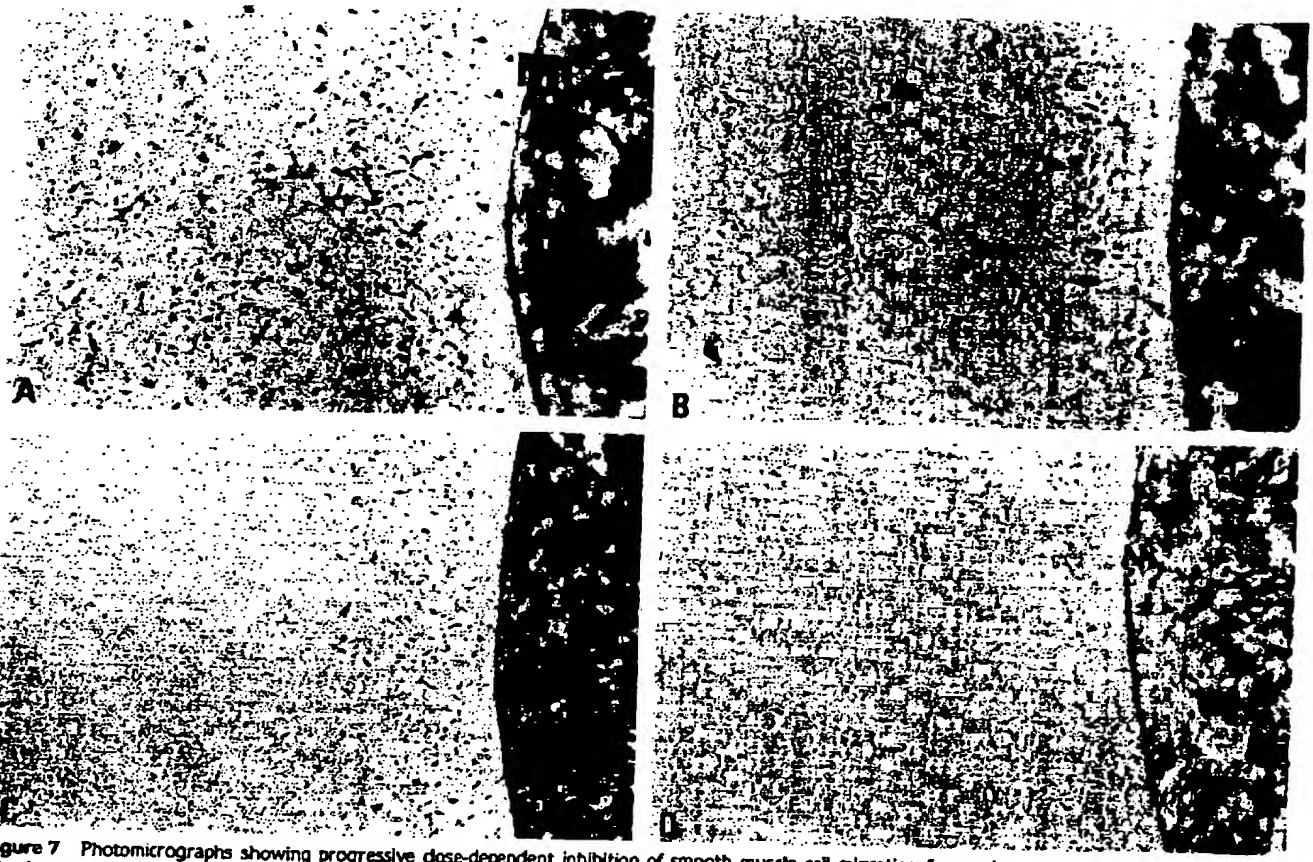

Figure 7 Photomicrographs showing progressive dose-dependent inhibition of smooth muscle cell migration from microscope coverslip on the 6th day of culture. Smooth muscle cells were stained with toluidine blue. **A** Control (collagen gel only); **B** type 1 collagen gel+1 mg/ml α-elastin; **C** type 1 collagen gel+5 mg/ml α-elastin; **D** type 1 collagen gel+10 mg/ml α-elastin. Original magnification, ×36.

tant changes in cell proliferation, as well as showing a primary effect on cell migration.

It was clear that smooth muscle cells are more sensitive than endothelial cells to the inhibitory effects of α-elastin on growth and migration. Receptor sites for elastin have been found on the membrane of smooth muscle cells, which may facilitate elastin control of their phenotype change, from synthetic to contractile type [12, 13]. The present results are consistent with the concept that the proliferation and migration of smooth muscle cells are regulated by elastin receptors locating on the cells' membrane. It is important to note that the effect of α-elastin was contained in solid phase collagen gel and may differ from that of pure or liquid α-elastin. These preliminary results suggest that incorporation of collagen gel containing α-elastin into the structure of an arterial prosthesis may inhibit intimal hyperplasia, without significantly affecting endothelium formation, an observation which may be useful in future prosthetic design.

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